

# Transgenic Plant Cells Lacking Mitochondrial Alternative Oxidase Have Increased Susceptibility to Mitochondria-Dependent and -Independent Pathways of Programmed Cell Death<sup>1</sup>

Christine A. Robson and Greg C. Vanlerberghe\*

Division of Life Sciences and Department of Botany, University of Toronto at Scarborough, 1265 Military Trail, Scarborough, Ontario, Canada M1C 1A4

The plant mitochondrial electron transport chain is branched such that electrons at ubiquinol can be diverted to oxygen via the alternative oxidase (AOX). This pathway does not contribute to ATP synthesis but can dampen the mitochondrial generation of reactive oxygen species. Here, we establish that transgenic tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) cells lacking AOX (AS8 cells) show increased susceptibility to three different death-inducing compounds (H<sub>2</sub>O<sub>2</sub>, salicylic acid [SA], and the protein phosphatase inhibitor cantharidin) in comparison with wild-type cells. The timing and extent of AS8 cell death are very similar among the three treatments and, in each case, are accompanied by the accumulation of oligonucleosomal fragments of DNA, indicative of programmed cell death. Death induced by H<sub>2</sub>O<sub>2</sub> or SA occurs by a mitochondria-dependent pathway characterized by cytochrome *c* release from the mitochondrion. Conversely, death induced by cantharidin occurs by a pathway without any obvious mitochondrial involvement. The ability of AOX to attenuate these death pathways may relate to its ability to maintain mitochondrial function after insult with a death-inducing compound or may relate to its ability to prevent chronic oxidative stress within the mitochondrion. In support of the latter, long-term treatment of AS8 cells with an antioxidant compound increased the resistance of AS8 cells to SA- or cantharidin-induced death. The results indicate that plants maintain both mitochondria-dependent and -independent pathways of programmed cell death and that AOX may act as an important mitochondrial "survival protein" against such death.

Programmed cell death (PCD) is an essential physiological process occurring during plant development and in response to biotic and abiotic stress (Beers and McDowell, 2001). An example is the hypersensitive response (HR), a rapid, localized cell death that occurs at sites of invasion by an incompatible pathogen and that acts to restrict the pathogen to the immediate area (Alvarez, 2000).

Although the cellular and molecular events involved in plant PCD are only beginning to be elucidated, the events involved in animal PCD (commonly called apoptosis) have been extensively characterized (for recent reviews, see Desagher and Martinou, 2000; Adams and Corey, 2001; Bratton and Cohen, 2001; Kaufmann and Hengartner, 2001). Animal PCD is primarily achieved by the activation of the Asp-specific Cys protease (caspase) cascade (Bratton and Cohen, 2001). Two types of pathways can lead to activation of this cascade. One pathway depends upon the participation of the mitochondrion, whereas the other pathway involves the interaction of a death receptor and

ligand. A key event in the mitochondrial pathway (and in some cases the receptor pathway as well) is release of the mitochondrial electron transport chain (ETC) protein cytochrome (*cyt c*) from the mitochondrion to the cytosol. *Cyt c* in the cytosol catalyzes the oligomerization of apoptotic protease activating factor-1. This promotes the activation of procaspase-9, which then activates procaspase-3, the most prevalent caspase in animal cells.

*Cyt c* release from the mitochondrion is tightly regulated by the Bcl-2 family of proteins. Anti-apoptotic Bcl-2 family members act to prevent *cyt c* release, whereas pro-apoptotic Bcl-2 family members promote such release (Adams and Corey, 2001). The actual mechanism(s) of *cyt c* release is still a matter of debate (Desagher and Martinou, 2000). Several distinct mechanisms are hypothesized, some of which involve rupture of the outer mitochondrial membrane, others of which result in the formation of pores in the membrane through which *cyt c* can pass.

There is also debate regarding the relative importance of caspase activation versus mitochondrial dysfunction in promoting animal PCD. Although it is apparent that changes in key mitochondrial parameters (such as membrane potential, rate of electron transport, rate of ATP synthesis, rate of reactive oxygen species [ROS] generation, and matrix Ca<sup>2+</sup> concentration) occur during death programs, the functional significance of these changes in terms of

<sup>1</sup> This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada and by a Premier's Research Excellence Award of Ontario (both to G.C.V.).

\* Corresponding author; e-mail gregv@utsc.utoronto.ca; fax 416-287-7642.

Article, publication date, and citation information can be found at [www.plantphysiol.org/cgi/doi/10.1104/pp.004853](http://www.plantphysiol.org/cgi/doi/10.1104/pp.004853).

promoting or attenuating cell death remains controversial (Green and Kroemer, 1998; Matsuyama and Reed, 2000; Mootha et al., 2001).

A prevalent theme in animal PCD research is that the intracellular redox state may play a critical role in the overall process (Voehringer et al., 2000; Kokoszka et al., 2001; Kowaltowski et al., 2001; Petrosillo et al., 2001). In this case, ROS generated by the mitochondrial ETC itself may be of particular significance. Such ROS might promote cell death via oxidative damage to the mitochondrion or by acting as signaling molecules in the death pathway.

It is unclear to what extent PCD events in plants are similar to those in animals. For example, although there is recent evidence for the release of cyt *c* from the mitochondrion during plant PCD (Sun et al., 1999; Balk and Leaver, 2001), bioinformatics analyses have not identified plant homologs of other key proteins in animal apoptosis such as Bcl-2 family members or caspases (Assaad, 2001). Nonetheless, expression of animal pro-apoptotic Bcl-2 family members in plants promotes PCD (Lacomme and Santa Cruz, 1999), whereas expression of anti-apoptotic members can suppress such death (Mitsuhashi et al., 1999). Also, there is biochemical evidence for caspase-like activity in plant cells (Sun et al., 1999; Korthout et al., 2000). Significantly, it was recently shown that plant mitochondria can display a permeability transition, with characteristics similar to that seen in animals (Arpagaus et al., 2002). Such a transition is implicated in many animal models of cyt *c* release (Desagher and Martinou, 2000). As a whole, recent studies suggest that the mitochondrion is an important player in at least some plant PCD programs (Jones, 2000; Lam et al., 2001).

The plant mitochondrion has unique components that alter mitochondrial function in comparison with animal cells and, hence, could alter the specific mechanisms by which the mitochondrion takes part in PCD. One such component is alternative oxidase (AOX), a mitochondrial inner membrane protein that functions as a part of the ETC, catalyzing the O<sub>2</sub>-dependent oxidation of ubiquinol, producing ubiquinone and water (Vanlerberghe and Ordog, 2002). Electron flow from ubiquinol to AOX is not coupled to the generation of proton motive force and, hence, is a nonphosphorylating branch of the ETC, bypassing the last two sites of energy conservation associated with the cyt pathway. Hence, AOX has important implications for mitochondrial function and cellular metabolism.

A few studies have implicated AOX in plant PCD. In *Arabidopsis*, a strategy used to identify genes induced early in the HR identified both AOX and a mitochondrial anion channel gene (Lacomme and Roby, 1999). The early inductions of these genes closely paralleled one another, were transient in nature, and were specific to an avirulent interaction. Mitochondrial anion channels are implicated to be

involved in the mechanism of cyt *c* release during animal apoptosis (Desagher and Martinou, 2000). What is unclear from this study is whether increased AOX expression was acting to attenuate or promote the death response. In another study, soybean (*Glycine max*) cells that had been given an anoxia pretreatment became more resistant to death caused by a subsequent challenge with H<sub>2</sub>O<sub>2</sub> (Amor et al., 2000). The anoxic pretreatment was associated with an increased level of AOX protein, and the protective effect of the pretreatment was suppressed by AOX inhibitors. Another interesting development is research implicating nitric oxide in the induction of the HR (Kleszig et al., 2000) because nitric oxide inhibits the plant cyt oxidase but not AOX (Miller and Day, 1996).

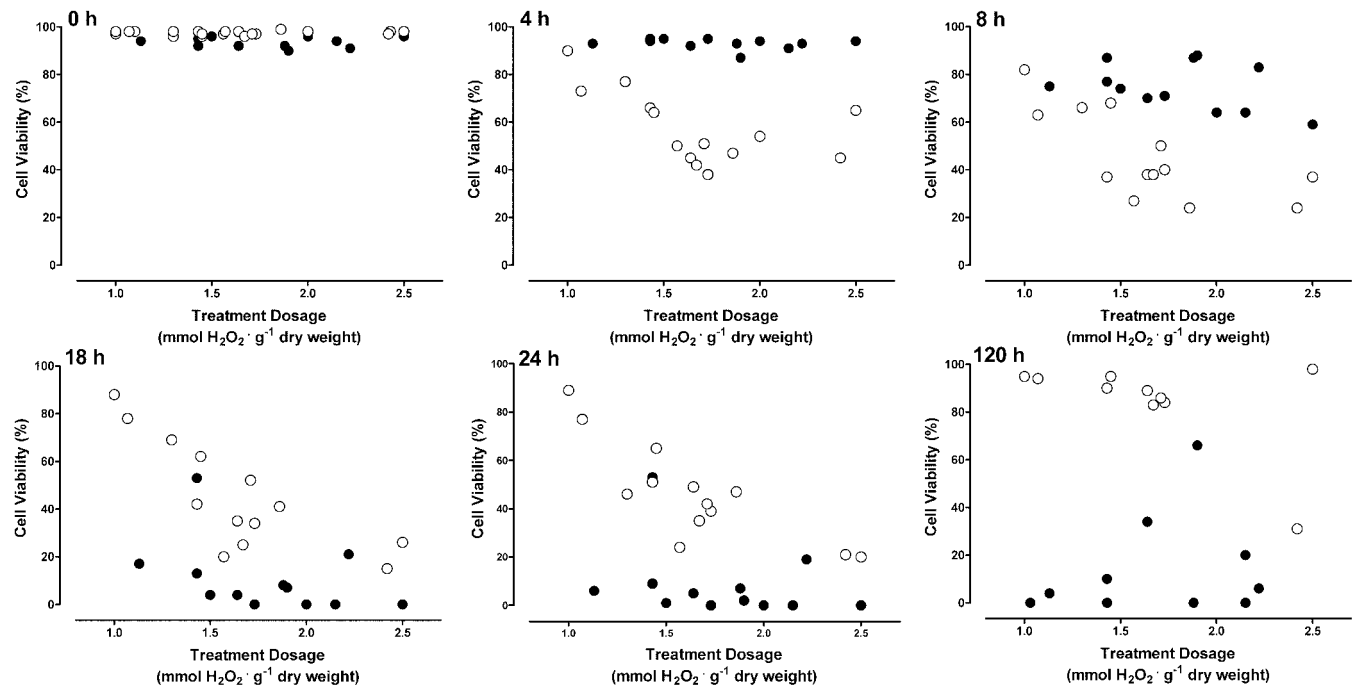
We previously generated transgenic tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) cells in which AOX gene expression was silenced by an antisense transgene (Vanlerberghe et al., 1994). Significantly, such cells have higher cellular levels of ROS than do wild-type (wt) cells (Parsons et al., 1999; Yip and Vanlerberghe, 2001), and the origin of these ROS is the mitochondrion (Maxwell et al., 1999). This data supports in organello evidence that AOX activity acts to dampen the generation of ROS by electron transport, presumably by preventing over-reduction of ETC components (Purvis, 1997). Here, we have used transgenic cells lacking AOX to investigate the potential role of this protein in plant PCD.

## RESULTS

### Antisense Cells Lacking Mitochondrial AOX Have Increased Susceptibility to Several Different Death-Inducing Compounds

When a low concentration of H<sub>2</sub>O<sub>2</sub> was added to the growth medium of wt tobacco cells (at 2 d after subculture), it resulted in a large (approximately 30%–60%) drop in culture viability within 4 h (Fig. 1). Culture viability dropped somewhat further by 8 and 18 h but then stabilized and began to recover so that by 120 h after treatment, the culture was again near 100% viable cells.

We compared the above wt response with that of transgenic tobacco cells (AS8) that lack mitochondrial AOX and found that the extent, pattern, and timing of cell death were much different. In the AS8 culture, there was no loss of culture viability at 4 h after treatment and only about a 10% to 30% drop by 8 h (Fig. 1). However, despite this apparent increased resistance to H<sub>2</sub>O<sub>2</sub> in the short term (0–8 h), we consistently found that between 8 and 18 h, AS8 culture viability dropped to at or near 0%. In most cases, there was no recovery of the culture by 120 h (Fig. 1). These differences in the pattern and extent of cell death between wt and AS8 cells were not attributable to differences in the dosage of H<sub>2</sub>O<sub>2</sub>



**Figure 1.** Viability of wt (○) and transgenic (AS8; ●) tobacco cell cultures at different times after the addition of  $\text{H}_2\text{O}_2$  (3–5 mM) to the culture medium at time 0. Each symbol represents data from an independent experiment.

(millimoles per gram dry weight of cells) applied (Fig. 1; see "Materials and Methods" for further explanation). The increased short-term resistance of AS8 cells to  $\text{H}_2\text{O}_2$  (0–8 h) may relate to the increased level of expression of antioxidant enzymes previously observed in these cells (Maxwell et al., 1999).

We compared the effects of  $\text{H}_2\text{O}_2$  with those of two other compounds, the plant phenolic salicylic acid (SA) and cantharidin, a potent inhibitor of Ser/Thr protein phosphatase types 1 and 2A (Li and Casida, 1992). Figure 2 shows the response of wt and AS8 cells to the addition of these compounds to the medium in comparison with the  $\text{H}_2\text{O}_2$  response. In the case of SA, neither culture showed significant loss of culture viability within the first 8 h (Fig. 2B). However, between 8 and 18 h, there was a complete loss of AS8 culture viability, just as was seen with  $\text{H}_2\text{O}_2$  (Fig. 2A). As an alternative, there was only a marginal loss of wt culture viability by 18 h. In the longer term, wt culture viability did drop further but only to a low of approximately 50%.

In the case of cantharidin, both wt and AS8 cultures again showed little loss of culture viability within the first 8 h (Fig. 2C). However, between 8 and 18 h, the AS8 culture again dropped to at or near 0%. In the wt, viability at 18 h was much higher (approximately 65%) and then decreased only gradually over the 120-h period.

#### Accumulation of Oligonucleosomal Fragments of DNA Indicates That AS8 Cells Undergo PCD

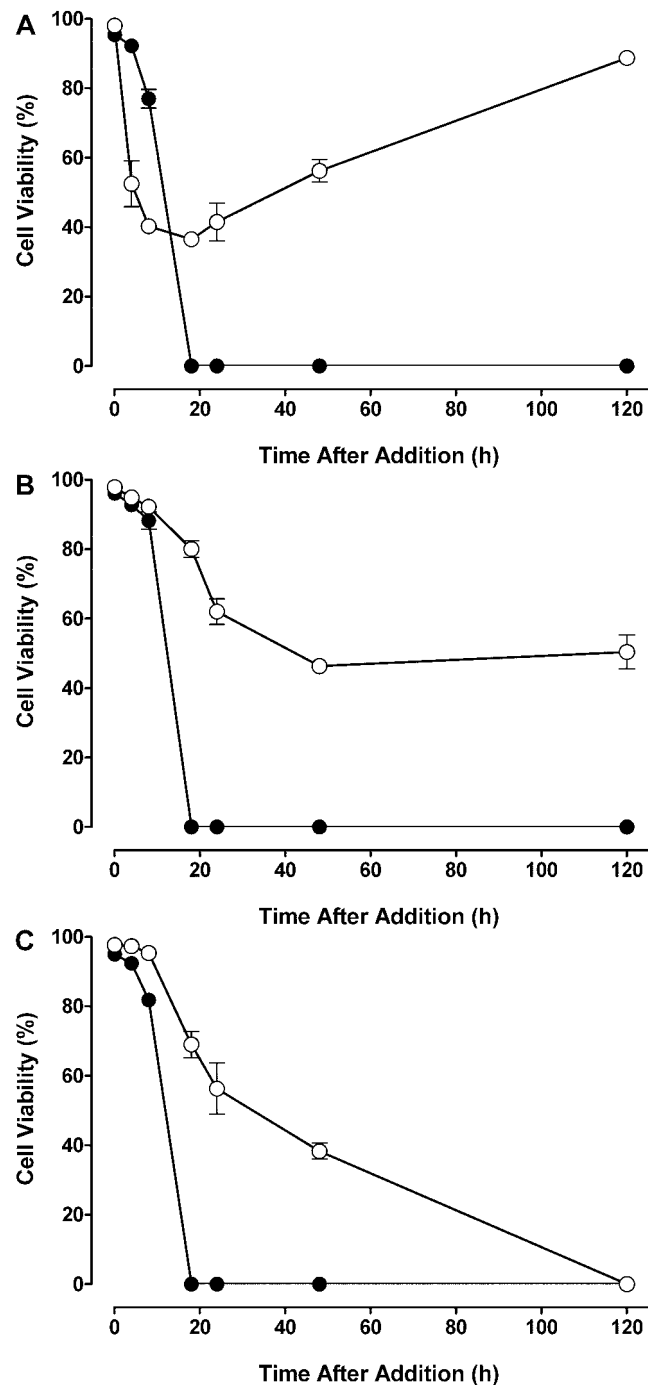
To determine whether cells were experiencing a necrotic or programmed form of cell death, we examined genomic DNA extracted from cells at different times (8, 24, 48, and 120 h) after addition of a death-inducing compound.

For AS8 cells treated with  $\text{H}_2\text{O}_2$ , we saw only high- $M_r$  DNA at 8 h (when viability of the culture is still high), but by 24 h, we observed the accumulation of lower  $M_r$  fragments of DNA (Fig. 3B). Comparison with a 100-bp DNA ladder indicated that the accumulating fragments matched the expected size of oligonucleosomal fragments (approximately 180–200 bp) and multiples thereof. This pattern (commonly called DNA laddering and indicative of PCD; Ryerson and Heath, 1996) was also readily visible at 48 and 120 h. In wt cells, no DNA laddering was observed in such experiments despite some cell death, particularly within the first 8 h after  $\text{H}_2\text{O}_2$  treatment (Fig. 3A).

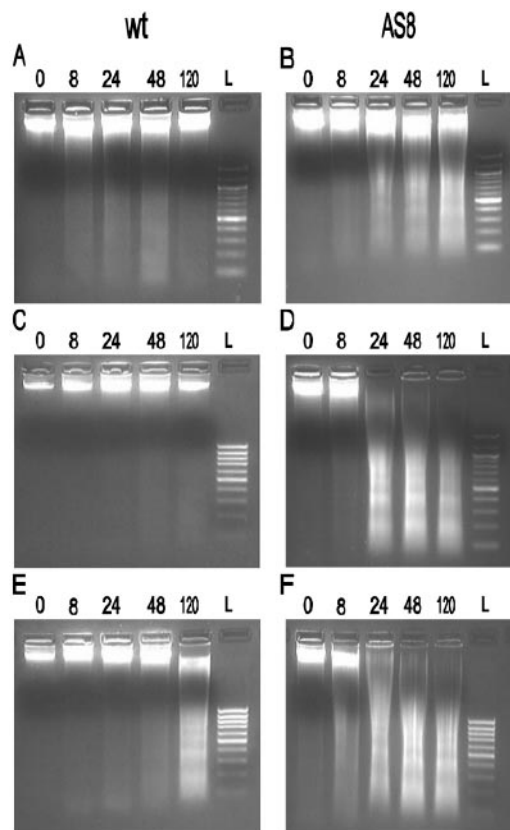
In the case of AS8 cells treated with SA, we readily observed DNA laddering at 24, 48, and 120 h (Fig. 3D). Also, interestingly, the loss of high- $M_r$  DNA in this case was much more complete than in  $\text{H}_2\text{O}_2$ -treated AS8 cells. As an alternative, for wt cells treated with SA, only high- $M_r$  DNA was observed throughout the 120-h period (Fig. 3C).

In the case of AS8 cells treated with cantharidin, DNA laddering was once again readily observed at 24, 48, and 120 h (Fig. 3F). For wt cells treated with

cantharidin, laddering was only observed in the long term (120 h, Fig. 3E), coincident with the large amount of cell death by this time point (see Fig. 2C).



**Figure 2.** Viability of wt (○) and transgenic (AS8; ●) tobacco cell cultures at different times after the addition of 3 mM H<sub>2</sub>O<sub>2</sub> (A), 500 μM SA (B), or 20 μM cantharidin (C) to the culture medium at time 0. These data are typical of the extent and timing of cell death in response to these treatments, as seen in many independent experiments. Data are the means ± SE from three to nine independent experiments. In some cases, error bars are smaller than the data symbols.



**Figure 3.** Agarose gel analysis of DNA isolated from tobacco cell cultures. DNA was isolated from either wt (A, C, and E) or AS8 (B, D, and F) cells at different times after the addition of 3 mM H<sub>2</sub>O<sub>2</sub> (A and B), 500 μM SA (C and D) or 20 μM cantharidin (E and F) to the culture medium at time 0. When present, DNA laddering was seen as the accumulation of up to four DNA bands averaging approximately 190, 400, 590, and 740 bp. All experiments were repeated at least twice, each time with similar results. Numbers refer to the number of hours of treatment before DNA isolation. L marks lanes containing a commercial 100-bp DNA ladder.

### PCD Is Preceded by Changes in Respiration and Mitochondrial Function

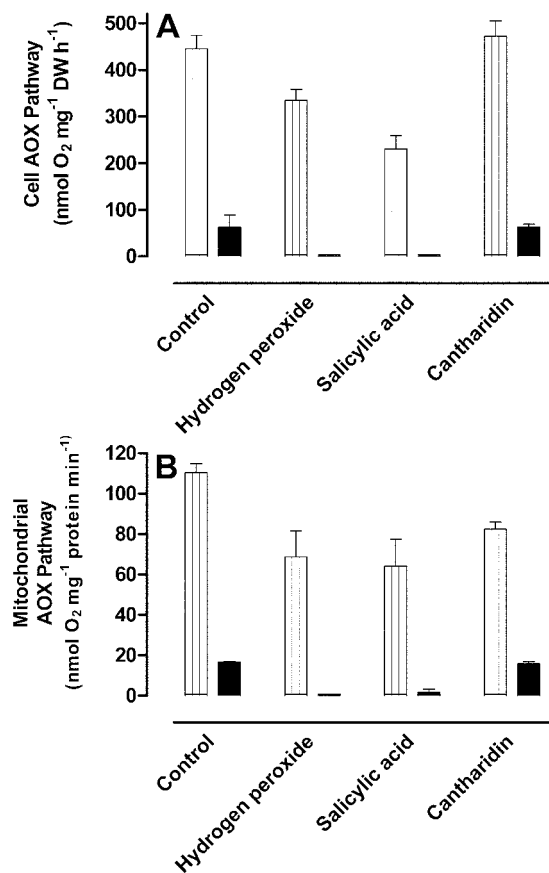
The respiratory characteristics of wt and AS8 cells were compared at 8 h after treatment with a death-inducing compound (i.e. before the major drops in viability). Also, we compared the respiratory characteristics being measured in whole cells (i.e. in vivo measurements) with those of mitochondria isolated from such cells (i.e. in organello measurements). This comparison was valuable in determining whether changes in whole-cell respiratory characteristics were the result of changes in mitochondrial ETC function specifically or the result of changes in upstream carbon metabolism. We also confirmed cell viability of the cultures for these experiments. In all cases, viability after the 8-h treatments was similar to that seen in Figure 2. That is, viability remained reasonably high (averaging from 82% to 97%, depending on the treatment) with the exception of wt cells treated with

H<sub>2</sub>O<sub>2</sub>, in which case culture viability had dropped to approximately 59%, as expected (see Figs. 1 and 2).

### AOX Capacity

In vivo and in organello assays were used to estimate the maximum potential rate of electron transport through the AOX pathway (see "Materials and Methods"). In the wt, AOX capacity in untreated (control) cells was high, whether measured in vivo (Fig. 4A) or in organello (Fig. 4B). An 8-h treatment of cells with H<sub>2</sub>O<sub>2</sub> or SA reduced this capacity somewhat (whether measured in vivo or in organello), but significant capacity was still maintained. Cantharidin treatment had no impact on in vivo AOX capacity, although it was reduced somewhat when measured in organello.

As expected, the AOX capacity of untreated (control) AS8 cells was very low in comparison with the wt, whether measured in vivo (Fig. 4A) or in organello (Fig. 4B). This indicates that the antisense



**Figure 4.** The AOX capacity of wt and transgenic (AS8) tobacco cells either left untreated (control) or treated for 8 h with 3 mM H<sub>2</sub>O<sub>2</sub>, 500 μM SA, or 20 μM cantharidin. AOX capacity of wt (hatched bars) and AS8 (black bars) were measured both in vivo (A) and in organello (B), as described in "Materials and Methods." Data are the means of two independent experiments, and error bars represent minimum and maximum values. Other data accompanying these experiments are presented in Figures 5 and 6.

transgene was effectively suppressing AOX expression in these cells. With H<sub>2</sub>O<sub>2</sub> or SA treatment, capacity was negligible either in vivo or in organello. As an alternative, in vivo or in organello AOX capacity after cantharidin treatment remained low and similar to untreated AS8 cells.

### Respiration

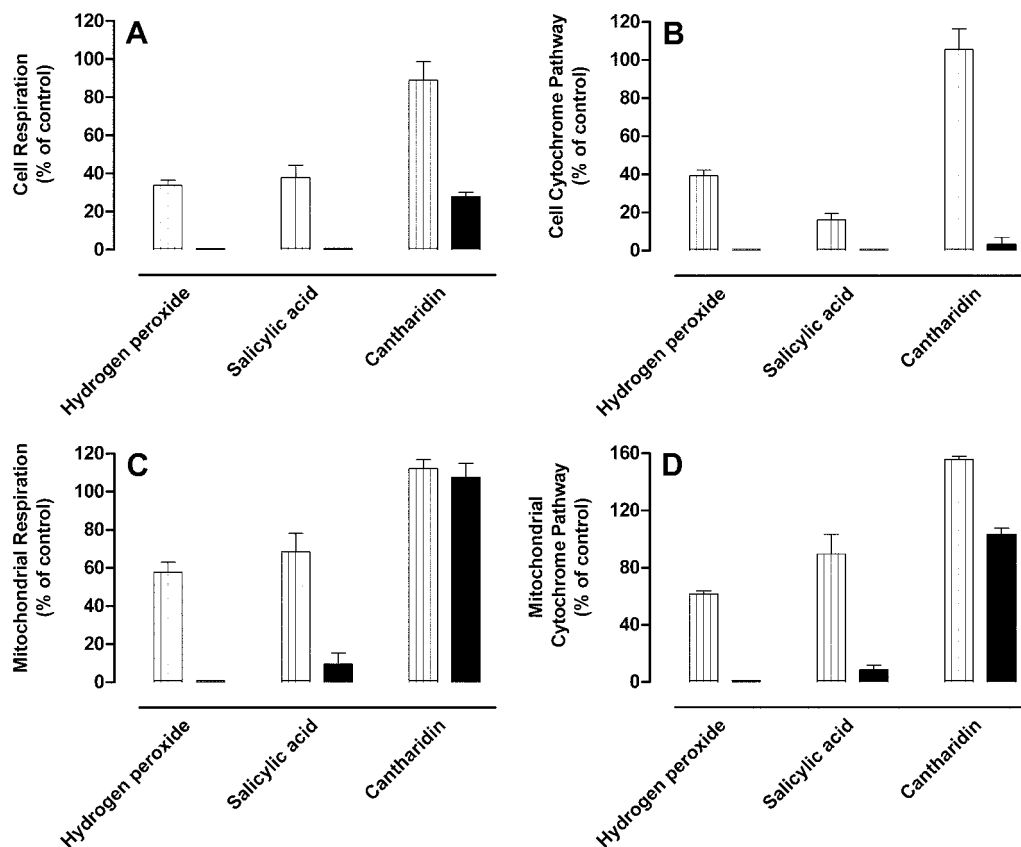
The respiration rate of whole cells (treated or untreated) was determined simply by monitoring oxygen uptake (in the absence of any inhibitors), whereas the respiration rate of isolated mitochondria was determined by monitoring oxygen uptake in the presence of a suite of respiratory substrates (see "Materials and Methods"). The in vivo data is, thus, a measure of actual cell respiratory activity, whereas the in organello data is a measure of the functional state of the mitochondrion.

The respiration rate of wt cells was reduced by approximately 60% at 8 h after treatment with H<sub>2</sub>O<sub>2</sub> or SA (Fig. 5A). In organello respiration was reduced by a similar extent (by approximately 40%) in response to these treatments (Fig. 5C). As an alternative, cantharidin-treated wt cells showed only a small decline in cell respiration and no decline (in fact a small increase) in mitochondrial respiration (Fig. 5, A and C).

The respiration of H<sub>2</sub>O<sub>2</sub>-treated AS8 cells was completely abolished, whether measured in vivo (Fig. 5A) or in organello (Fig. 5C). Similarly, SA-treated AS8 cells showed no cell respiration, although a low level of mitochondrial respiration (<10% of control) was still observed. Interestingly, cantharidin reduced in vivo AS8 respiration to less than 30% of control rates (Fig. 5A), but this occurred in the absence of any effect on mitochondrial respiration, which remained at levels slightly greater than control values (Fig. 5C). These data indicate that cantharidin treatment was reducing the respiratory activity of AS8 cells but that this was not attributable to a direct loss of mitochondrial function.

### Cyt Pathway Capacity

In vivo and in organello assays were used to estimate the functional state of the cyt pathway (see "Materials and Methods"). In wt cells, treatment with H<sub>2</sub>O<sub>2</sub> or SA reduced cyt path capacity measured in either cells or mitochondria to a similar extent as respiration was reduced (compare Fig. 5, B and D with A and C). As an alternative, no loss of cyt path (in vivo or in organello) was seen with cantharidin, similar to what had been noted for the respiration of cantharidin-treated wt cells. In fact, the mitochondrial cyt path capacity of cantharidin-treated cells actually increased nearly 1.6-fold in comparison with untreated wt cells (Fig. 5D).



**Figure 5.** The respiration and cyt pathway capacity of wt and transgenic (AS8) tobacco suspension cells after an 8-h treatment with 3 mM H<sub>2</sub>O<sub>2</sub>, 500 μM SA, or 20 μM cantharidin. Respiration (A and C) and cyt capacity (B and D) were measured in vivo (A and B) and in organello (C and D) for both wt (hatched bars) and AS8 (black bars) as described in "Materials and Methods." Data are the means of two independent experiments, and error bars represent minimum and maximum values. Cell respiration of control (untreated) cells averaged 776 (wt) and 511 (AS8) nmol O<sub>2</sub> mg<sup>-1</sup> dry weight h<sup>-1</sup>. Mitochondrial respiration of control (untreated) cells averaged 216 (wt) and 81 (AS8) nmol O<sub>2</sub> mg<sup>-1</sup> protein min<sup>-1</sup>. Cell cyt pathway of control (untreated) cells averaged 1,289 (wt) and 1,044 (AS8) nmol O<sub>2</sub> mg<sup>-1</sup> dry weight h<sup>-1</sup>. Mitochondrial cyt pathway of control (untreated) cells averaged 102 (wt) and 69 (AS8) nmol O<sub>2</sub> mg<sup>-1</sup> protein min<sup>-1</sup>. Other data accompanying these experiments are presented in Figures 4 and 6.

In AS8, both the cell and the mitochondrial cyt path were completely abolished after an 8-h treatment with H<sub>2</sub>O<sub>2</sub> or SA, except for a small amount of the mitochondrial cyt path in the SA-treated cells (Fig. 5, B and D). In the case of cantharidin, the AS8 cell cyt path was almost abolished, despite no loss of the mitochondrial cyt path (Fig. 5, B and D).

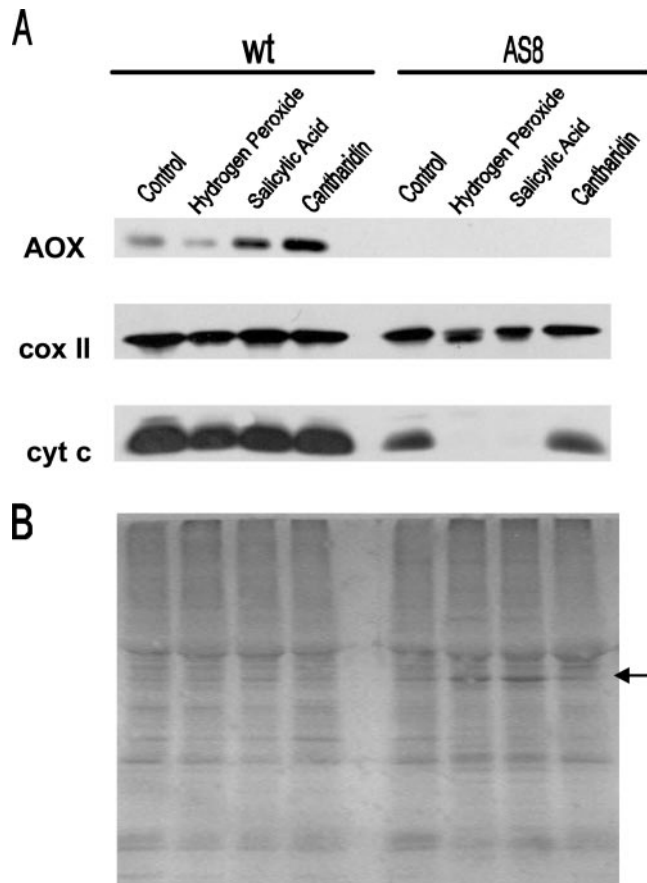
#### In Antisense Cells, the Loss of Mitochondrial Function after H<sub>2</sub>O<sub>2</sub> or SA Treatment and Preceding PCD Involves Cyt *c* Release from the Mitochondrion

We examined the level of ETC proteins in mitochondria isolated after the 8-h treatments with a death-inducing compound. In mitochondria from wt cells, AOX protein was always visible in the mitochondria from both treated and untreated cells, consistent with the presence of AOX capacity in these cells (Fig. 6A). As an alternative, we were unable to detect any AOX protein in mitochondria isolated

from AS8 cells, which is consistent, in these cases, with the lack of AOX capacity (Fig. 6A).

The levels of two proteins in the cyt pathway were also examined. In the wt, cyt *c* was present in mitochondria from both untreated cells and cells treated with H<sub>2</sub>O<sub>2</sub>, SA, or cantharidin (Fig. 6A). However, in the case of AS8 cells, we found that both the H<sub>2</sub>O<sub>2</sub> and SA treatments resulted in a massive loss of cyt *c* protein from the mitochondrion. As an alternative, cantharidin treatment did not generate this loss of cyt *c*. Cyt oxidase subunit II (cox II) was abundant in mitochondria from both wt and AS8 cells, whether untreated or treated with one of the death-inducing compounds (Fig. 6A).

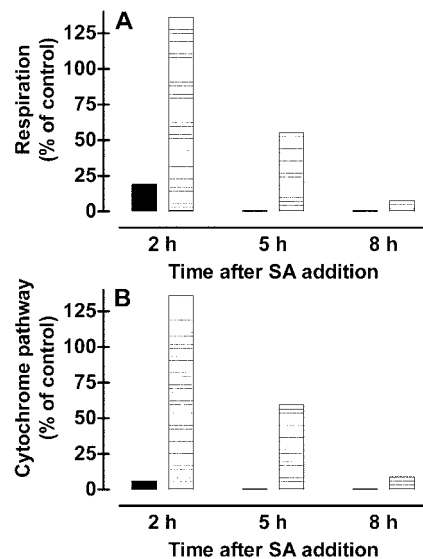
Figure 6B is an example of a Coomassie-stained SDS-PAGE gel similar to that used for the immunoblot analyses in Figure 6A. We consistently found that in the AS8 cells treated for 8 h with either H<sub>2</sub>O<sub>2</sub> or SA (i.e. the two treatments that result in cyt *c* release from the mitochondrion; Fig. 6A), there were



**Figure 6.** Protein analysis of mitochondria isolated from wt and AS8 cells. Cells had been left untreated (control) or treated for 8 h with 3 mM H<sub>2</sub>O<sub>2</sub>, 500 μM SA, or 20 μM cantharidin before mitochondrial isolation. Mitochondrial protein (50 μg) was separated by reducing SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies raised to AOX, cox II, or cyt c. Results of this immunoblot analysis are shown in A. B, A typical Coomassie-stained SDS-PAGE gel, with lanes corresponding to those in A. Note that an unidentified protein of approximately 60 kD (indicated by the arrow in Fig. 6B) was the increased intensity of an unidentified protein of approximately 60 kD.

several changes in the mitochondrial protein profile, and these changes always occurred similarly in both of these treatments and in none of the other treatments. One prominent change (marked by the arrow in Fig. 6B) was the increased intensity of an unidentified protein of approximately 60 kD.

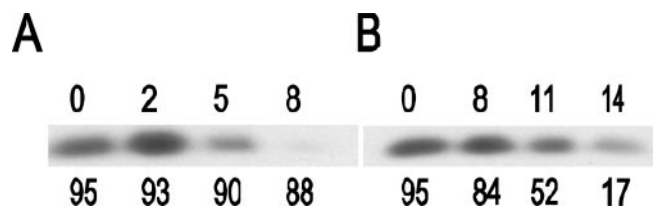
A time-course experiment examined the effects of SA on AS8 cells in more detail. We found that AS8 cell respiration and cell cyt pathway capacity were severely depressed within 2 h after addition of SA, despite no loss of mitochondrial respiration or mitochondrial cyt path at this time point (Fig. 7). This indicates that a general depression of cell respiration precedes changes in mitochondrial function. By 5 h, cell respiration and cell cyt path were completely lost. By this time as well, mitochondrial respiration



**Figure 7.** Respiration and cyt pathway capacity of transgenic (AS8) cells at different times after the addition of 500 μM SA at time 0. Respiration (A) and cyt pathway capacity (B) were determined in vivo (black bars) and in organello (hatched bars), as described in “Materials and Methods.” In control (untreated) AS8 cells, cell respiration was 571 nmol O<sub>2</sub> mg<sup>-1</sup> dry weight h<sup>-1</sup>, mitochondrial respiration was 75 nmol O<sub>2</sub> mg<sup>-1</sup> protein min<sup>-1</sup>, cell cyt pathway was 1,040 nmol O<sub>2</sub> mg<sup>-1</sup> dry weight h<sup>-1</sup> and mitochondrial cyt pathway was 68 nmol O<sub>2</sub> mg<sup>-1</sup> protein min<sup>-1</sup>.

and mitochondrial cyt path were each reduced to about 50% of control levels (Fig. 7), and this correlated with some loss of cyt c from the mitochondrion by this time (Fig. 8A). Finally, at 8 h, mitochondrial respiration and cyt path were almost completely lost (Fig. 7), as was the level of cyt c (Fig. 8A). Importantly, all of these events precede any significant loss of culture viability (Fig. 8A).

To examine whether cyt c loss might still be an event preceding the death of cantharidin-treated AS8 cells (despite the lack of cyt c release by 8 h, Fig. 6A), a time-course experiment examined viability and cyt c level in the longer term. By 11 and 14 h after cantharidin treatment, viability dropped to 52% and 17%, respectively (Fig. 8B). Despite these now large drops in viability, cyt c remained abundant in iso-



**Figure 8.** Culture viability and the level of cyt c in mitochondria isolated from AS8 cells at different times after treatment with 500 μM SA (A) or 20 μM cantharidin (B). The upper row of numbers refers to the number of hours of treatment before mitochondrial isolation. The bottom row of numbers refers to the percentage of viable cells in the culture just before mitochondrial isolation.

lated mitochondria (Fig. 8B), and cyt path capacity was still evident (data not shown).

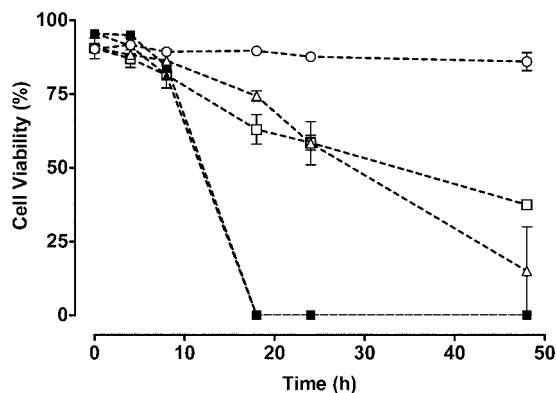
#### Long-Term Pretreatment of Antisense Cells with Antioxidants Attenuates PCD

We tested whether the antioxidant compound flavone would delay or prevent PCD in AS8 cells. In one type of experiment, 100  $\mu\text{M}$  flavone was added to the cell culture 15 min before the addition of either SA or cantharidin. This short-term pretreatment with flavone had no significant effect on either the timing or extent of cell death in response to either SA or cantharidin (data not shown).

In another type of experiment, cells were grown for 2 d (i.e. from the time of subculture) in the presence of flavone before the addition of a death-inducing compound. This long-term pretreatment was found to significantly delay the death induced by either SA or cantharidin (Fig. 9). The treatments in which death was delayed still displayed DNA laddering (indicative of PCD), except that the laddering was delayed because of the slower death of the culture (data not shown).

#### DISCUSSION

Two key findings of the present research are as follows: (a) Transgenic cells lacking AOX are more susceptible than wt cells to PCD. To our knowledge, this is the first example showing that manipulation of a component of plant mitochondrial metabolism can dramatically influence PCD. Our discussion of this comparison between wt and transgenic cells and our hypotheses regarding the increased susceptibility of AS8 cells will be discussed later. (b) Using



**Figure 9.** The effect of flavone on SA- and cantharidin-induced death of AS8 cells. When present, flavone (100  $\mu\text{M}$ ) was added to cell cultures 2 d before time 0. When present, SA (500  $\mu\text{M}$ ) and cantharidin (20  $\mu\text{M}$ ) were added to cell cultures at time 0. Treatments were as follows: flavone only (○); SA only (▲); cantharidin only (■); SA + flavone (△); and cantharidin + flavone (□). Note that the black triangles are being obscured by the black squares at most time points. Data are the means  $\pm$  SE from three independent experiments. In some cases, error bars are smaller than the data symbols.

different death-inducing compounds, we have identified two different pathways of PCD, one pathway with an apparent mitochondrial connection, and a second pathway lacking any obvious mitochondrial involvement.

#### Mitochondria-Dependent and -Independent Pathways of PCD in Tobacco Cells

The discussion below pertains to AS8 cells, in which PCD was readily induced.  $\text{H}_2\text{O}_2$ , SA, and cantharidin each induced PCD in AS8, and the timing and extent of death were remarkably similar between the three treatments (Figs. 1 and 2). In each case, a major drop in culture viability occurred between 8 and 18 h after treatment. DNA laddering became apparent by 24 h after each treatment and then persisted through 120 h (Fig. 3). Despite these similarities between treatments, analyses of cellular events preceding death (i.e. within the first 8 h) indicated that the effects of  $\text{H}_2\text{O}_2$  and SA were very similar to one another but that the effects of cantharidin were fundamentally different.

Examination of AS8 respiratory characteristics in response to the different death-inducers revealed a striking similarity between the effects of  $\text{H}_2\text{O}_2$  and SA. Both compounds abolished the capacity for cyt path respiration by 8 h after treatment. This was confirmed in both cells and mitochondria derived from such cells (Fig. 5, B and D). As a result of this inhibition (and the lack of AOX in AS8), neither cells nor mitochondria displayed any respiratory  $\text{O}_2$  uptake (Fig. 5, A and C). Importantly, the lack of cyt path at 8 h was accompanied by an almost complete loss of cyt *c* from the mitochondrion (Fig. 6).

To examine the above changes more closely, a time-course experiment was performed using SA. Two important observations arose from these experiments. First, cell respiration was dramatically depressed at 2 h after treatment, but this was clearly not due to an inhibition of mitochondrial ETC function. Mitochondrial ETC function appeared normal, whether evaluated using the mitochondrial respiration or mitochondrial cyt path assay (Fig. 7). Note that the cell cyt path assay did indicate a low cyt path capacity at 2 h. However, this is artifactual, arising from the fact that cell respiration had been dramatically reduced by this time point. These results illustrate the importance of having done measurements of both cell and mitochondrial respiratory characteristics. Examining both determined whether changes in cell respiratory characteristics were due to changes in mitochondrial ETC function or some other event.

The other important observation arising from the time-course experiment was that the change in mitochondrial cyt path capacity approximately correlated with the change in cyt *c* level in the mitochondrion (Figs. 7B and 8A). At 2 h after treatment, no loss of cyt path capacity or cyt *c* level was obvious, whereas



at 5 h, both parameters began to decline. By 8 h, both cyt path capacity and cyt *c* level were very low. Importantly, these events precede any significant level of cell death in the culture.

In summary, the results show the following order of events in SA-treated AS8 cells: (a) a severe depression of whole-cell respiratory activity before any change in mitochondrial ETC function (between approximately 0 and 2 h after treatment); (b) a rapid decline in mitochondrial ETC function in which a loss of cyt *c* from the mitochondrion may be a critical event (between approximately 5 and 8 h after treatment); and (c) rapid PCD (between 8 and 18 h after treatment).

SA and H<sub>2</sub>O<sub>2</sub> were chosen as death inducers for our experiments because each has been implicated to play a role in the HR (Jabs, 1999; Alvarez, 2000) and other examples of PCD (Morris et al., 2000; Bethke and Jones, 2001; Rao and Davis, 2001). These compounds likely play an important role as signaling molecules during the HR, but their mechanism(s) of action remains unclear. Here, we find that both compounds can induce a mitochondrial pathway of PCD, characterized by cyt *c* loss from the mitochondrion. These results suggest that the mitochondrion may play a central role in PCD pathways involving SA or H<sub>2</sub>O<sub>2</sub>. Interestingly, acetylsalicylic acid (aspirin, an SA derivative) can induce animal apoptosis, also by a mitochondrial pathway involving cyt *c* release (Pique et al., 2000).

We were struck by how similar the response of AS8 cells were to SA and H<sub>2</sub>O<sub>2</sub>. Both compounds induced almost identical changes in respiratory characteristics and induced cyt *c* release. Also, both compounds generated several changes in the mitochondrial protein profile of AS8 cells, and these changes were identical between the two treatments (Fig. 6B). Attempts are under way to identify these proteins, with the hope that it will provide insight into the plant mitochondrial PCD pathway. The similarity between the SA and H<sub>2</sub>O<sub>2</sub> treatments is consistent with evidence that these compounds may act as complementary or synergistic signals during the HR and in response to abiotic stress (Jabs, 1999; Alvarez, 2000).

Cantharidin treatment had a different effect than H<sub>2</sub>O<sub>2</sub> and SA on AS8 respiratory characteristics. Cell respiration and apparent cell cyt pathway capacity were dramatically reduced at 8 h (just as seen with the other treatments), but these changes in cell respiratory characteristics were not attributable to changes in mitochondrial ETC function. Mitochondrial ETC function at 8 h (whether assessed by the mitochondrial respiration or mitochondrial cyt pathway capacity assay) was similar to that of mitochondria from the untreated cells (Fig. 5). Consistent with this, these mitochondria retained their cyt *c* (Fig. 6A).

A longer term time-course experiment with cantharidin (8, 11, and 14 h) clearly showed that cyt *c*

loss was not an event preceding death of the culture. For example, at 11 h after cantharidin treatment, 48% of the cells were already dead but high levels of cyt *c* were still evident (Fig. 8B). Some loss of cyt *c* appeared to occur at the later time points (particularly at 14 h), but this is probably attributable to a general decline in cell organization, because culture viability by this time point had already dropped to only 17%.

In summary, the results suggest the following order of events in cantharidin-treated AS8 cells: (a) a depression of whole-cell respiratory activity (within 8 h), before any change in mitochondrial ETC function; and (b) rapid PCD (between 8 and 18 h), before any apparent cyt *c* release from the mitochondrion. Previous studies also found that addition of Ser/Thr protein phosphatase inhibitors induced rapid plant cell death, suggesting that changes in protein phosphorylation may be a common event in death induction (Suzuki et al., 1999; Rakwal et al., 2001).

Despite no indication that the mitochondrion is involved in the cantharidin-induced pathway (at least with respect to cyt *c* release), it is interesting that cantharidin severely depressed whole-cell respiration in AS8 cells, just as was found with H<sub>2</sub>O<sub>2</sub> and SA. This may be coincidental or may indicate that a depression of whole-cell respiration is a common early event among these different PCD pathways. The ability of SA to dramatically inhibit whole-cell respiration in the short term (30 min) but without any apparent effect on mitochondrial ETC function was also seen previously (Xie and Chen, 1999). That study did not examine whether a longer term treatment would have eventually led to mitochondrial cyt *c* release and cell death.

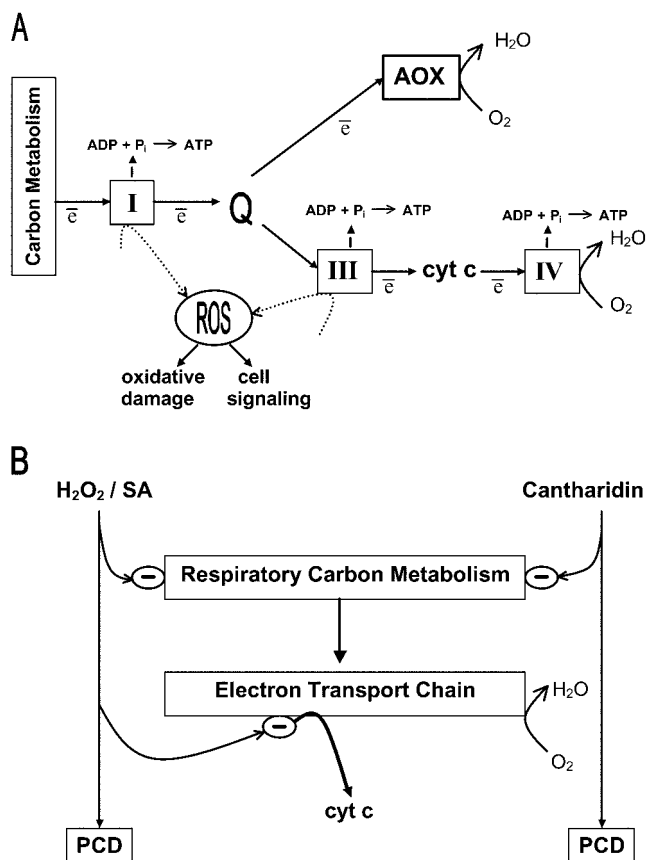
#### How Does AOX Influence Susceptibility to PCD?

An important challenge in future experiments is to determine why antisense cells lacking AOX are more susceptible to PCD-inducing compounds than wt cells with abundant AOX. Such a determination could provide insight into the cellular conditions that promote cell survival versus those promoting cell death. The usefulness of such an approach is illustrated by experiments using an apoptosis-sensitive and -resistant murine B-cell lymphoma model system (Voehringer et al., 2000). This study compared gene expression profiles of sensitive and resistant lines, both before and after a death-inducing treatment. As an alternative, the barley (*Hordeum vulgare*) aleurone has provided a unique system to study sensitivity to PCD in plants. In this case, sensitivity is hormonally controlled by gibberellic acid and abscisic acid (Bethke and Jones, 2001). Significantly, each of the above studies implicated ROS and intracellular redox state as a key variable in sensitivity to PCD.

Our work shows that tobacco cells can undergo PCD by at least two different pathways: a mitochondria-dependent pathway involving cyt *c* release and a

pathway without any obvious mitochondrial involvement (Fig. 10B). Nonetheless, the AS8 cells with altered mitochondrial function (i.e. a lack of AOX) have increased susceptibility to compounds inducing either of these PCD pathways. Below, we describe our two broad hypotheses as to why wt

and AS8 cells differ in their susceptibility to PCD. Although discussed separately, the two hypotheses are not mutually exclusive, and it seems possible that both may play a role or that their relative importance may depend upon whether the mitochondria-dependent or -independent pathway is considered.



**Figure 10.** A, The mitochondrial ETC transfers electrons from carbon oxidation to O<sub>2</sub>, producing water. ETC complexes I, III, and IV generate proton motive force used to produce ATP via ATP synthase (not shown). When some components in the ETC become more highly reduced, single-electron reduction of O<sub>2</sub> gives rise to superoxide and subsequently other ROS. The ROS may act as important signaling molecules in the cell but in excess may also cause oxidative damage. The plant ETC is branched at ubiquinone (Q) so that electrons can pass to O<sub>2</sub> via AOX, thus bypassing complex III, cyt *c*, and complex IV. This provides a potential mechanism to (a) modulate the rate of ATP production; (b) maintain electron transport when other downstream ETC components or ADP are limiting; and (c) modulate the reduction state of ETC components, thus, regulating the rate of ROS generation. B, Pathways of PCD in tobacco cells. H<sub>2</sub>O<sub>2</sub> or SA induce a mitochondria-dependent pathway in which cyt pathway function is disabled by cyt *c* release. Cantharidin induces a mitochondria-independent pathway not dependent upon cyt *c* release. Both pathways cause an early general depression of whole-cell respiratory activity, the occurrence of which is independent of mitochondrial dysfunction, and both pathways result in a similar extent and timing of cell death and DNA laddering. Antisense cells lacking AOX have increased susceptibility to both PCD pathways. This may relate to the ability of AOX to modulate mitochondrial function or to prevent chronic oxidative stress in the cell.

#### Mitochondrial Function Hypothesis

In this case, it is the activity of AOX in wt cells after the insult with a death-inducer that is primarily responsible for the differential sensitivity of wt and AS8 cells to PCD. In other words, after the insult of wt cells with a death-inducing treatment, active participation of AOX in the respiration of these cells acts to attenuate cell death in some fashion. Such a hypothesis is dependent upon wt cells maintaining a functional AOX pathway after the treatments, which is what we observed. After an 8-h treatment with H<sub>2</sub>O<sub>2</sub>, SA, or cantharidin, significant levels of AOX capacity were present, whether measured in cells or mitochondria (Fig. 4).

A characteristic of AOX activity is that electron flow from ubiquinol to oxygen is uncoupled from the generation of proton motive force (Fig. 10A). This, combined with the ability to biochemically regulate electron flux to AOX in a sophisticated manner (Vanlerberghe et al., 1998), provides the mitochondrion with considerable metabolic flexibility. This flexibility could potentially allow the mitochondrion to (a) modulate the rate of ATP production; (b) maintain electron flux to oxygen when other downstream ETC components or ADP are limiting; or (c) modulate the reduction state of ETC components, thereby controlling the rate of generation of ROS by electron transport (Fig. 10A). Significantly, mitochondrial parameters such as these are commonly implicated as important during animal PCD, although the role of individual parameters in promoting or attenuating important mitochondrial events such as cyt *c* release remains unclear (see introduction).

If AOX activity in wt cells acts to attenuate PCD in response to SA or H<sub>2</sub>O<sub>2</sub>, it seems probable that it would have to do so by promoting, either directly or indirectly, the maintenance of cyt *c*. In plants, nothing is known of the mechanisms responsible for cyt *c* loss, making it difficult to speculate on a mechanism by which AOX activity might attenuate this event.

In animals, the Bcl-2 family of proteins tightly regulates the cyt *c* release associated with PCD (Adams and Corey, 2001). Such active regulation is critical so that PCD is not inappropriately triggered or suppressed. As it becomes more firmly established that cyt *c* release is also an important event in plant PCD, the question arises as to how the process is actively regulated, because bioinformatics analyses have not revealed the presence of Bcl-2-like proteins in plants. If plants do lack a Bcl-2-like regulatory system, it

seems that some other regulatory mechanism would be required. Our work here suggests that the abundance and activity of AOX may serve such a regulatory function.

### *Chronic Oxidative Stress Hypothesis*

In this case, it is the absence of AOX in AS8 cells before the insult with a death inducer that is primarily responsible for the differential sensitivity of wt and AS8 cells to PCD. An important consequence of the lack of AOX in AS8 is that these cells experience constitutive higher *in vivo* levels of ROS than do wt cells (Maxwell et al., 1999; Parsons et al., 1999; Yip and Vanlerberghe, 2001). It has been elegantly shown that the source of these increased ROS is the mitochondrion (Maxwell et al., 1999) and that this is accompanied by changes in the expression of nuclear genes encoding antioxidant enzymes (such as catalase) and other genes known to respond to increased ROS, such as PR-1 (Maxwell et al., 1999). Such results clearly indicate that the increased mitochondrial ROS being produced in AS8 cells is physiologically relevant, having altered the pattern of gene expression in these cells.

Mitochondrial oxidative damage and changes in gene expression attributable to oxidative stress are widely implicated as important contributors to many PCD pathways in animal and plant cells (for review, see Jabs, 1999; see introduction). It seems possible that the chronic increased level of mitochondrial ROS ensures that AS8 cells are more "competent to die" than are wt cells. In plants, we are only beginning to identify genes that may play a role in PCD (Lacomme and Roby, 1999; Pontier et al., 1999; Hoerberichts et al., 2001). It will be of interest whether the expression of such candidate genes differs between wt and AS8 cells. It will also be of interest to determine whether AS8 mitochondria show signs of increased oxidative damage in comparison with wt cells.

Results of experiments using the antioxidant flavone are consistent with the chronic oxidative stress hypothesis. Flavone was used in these experiments because it has previously been shown to reduce the *in vivo* level of ROS in these plant cells (Maxwell et al., 1999). If the increased susceptibility of AS8 cells to PCD is due to oxidative damage and/or to changes in gene expression resulting from chronic oxidative stress, then one might expect that a short-term treatment with flavone (i.e. the 15-min treatment) would be insufficient time to remedy this and, hence, insufficient time to alter the cell death pattern. This is what we observed. This experiment also illustrates that the presence of flavone during treatment with SA or cantharidin is, in itself, insufficient to alter the death pattern. As an alternative, a long-term treatment with flavone (i.e. the 2-d treatment; Fig. 9) would allow sufficient time for oxidative damage and/or gene expression patterns (and, hence, the cell

death pattern) to be altered. Again, this is what we observed (Fig. 9).

Together, the above results are consistent with the hypothesis that chronic oxidative stress is "priming" AS8 cells for PCD and that if this stress is relieved (such as with flavone), the cells become less susceptible to PCD. With this view, the importance of AOX in susceptibility to PCD lies in its ability to continually dampen the mitochondrial generation of ROS, hence preventing oxidative damage, aberrant gene expression, or some other consequence of increased ROS that favors PCD (Fig. 10A).

## MATERIALS AND METHODS

### Plant Material, Growth Conditions, and Experimental Treatments

The cells used were derived from leaves of wt and transgenic tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) and were in culture for approximately 8 years before this study (Vanlerberghe et al., 1994). The transgenic cells (AS8) constitutively express an antisense construct of the nuclear gene *Aox1*, encoding a tobacco AOX. Hence, the normal expression of AOX in these cells is severely impaired.

Cell cultures (200 mL of culture in 500-mL Erlenmeyer flask) were grown in the dark on a rotary shaker (140 rpm) at 28°C and were subcultured every 7 d by dilution in fresh growth medium. The growth medium (Linsmaier and Skoog, 1965) contains 3% (w/v) Suc as carbon source. Experiments were always initiated using cells at 2 d after subculture. All experimental compounds to be added to cultures (cantharidin, flavone, H<sub>2</sub>O<sub>2</sub>, and SA) were from Sigma-Aldrich Canada (Oakville, ON, Canada). In each case, stock solutions were made fresh the day of use and filter-sterilized when required.

We were careful to ensure that our subculturing routine was generating wt and AS8 cultures that, at 2 d after subculture, were of similar density to one another and of similar density from one experiment to the next. In initial experiments, we both determined culture density just before H<sub>2</sub>O<sub>2</sub> addition and varied the initial dosage of H<sub>2</sub>O<sub>2</sub> applied (i.e. millimoles of H<sub>2</sub>O<sub>2</sub> per gram dry weight of cells; Fig. 1). These analyses showed that the differences seen between wt and AS8 cultures in the extent of cell death were not due to differences in culture density at the time of H<sub>2</sub>O<sub>2</sub> addition. Similar conclusions were drawn for the SA and cantharidin treatments.

### Respiratory Characteristics of Cells

Suspension cells (1–2.5 mg dry weight mL<sup>-1</sup> in their culture medium) were placed in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 28°C. Once a steady control rate of O<sub>2</sub> uptake was established (after 1–3 min), other additions were made to the cuvette. An uncoupler of oxidative phosphorylation (1 μM *p*-trifluoromethoxycarbonyl-cyanide) and inhibitors of cytochrome oxidase (1 mM KCN) and AOX (20 μM *n*-propyl gallate) were used. Under these conditions, cell respiration is defined as the O<sub>2</sub> uptake in the absence of any additions minus any residual respiration (O<sub>2</sub> uptake in the presence of both KCN and *n*-propyl gallate). Cell cytochrome pathway capacity is defined as O<sub>2</sub> uptake in the presence of *p*-trifluoromethoxycarbonyl-cyanide and *n*-propyl gallate that was sensitive to KCN. Cell AOX pathway capacity is defined as the O<sub>2</sub> uptake in the presence of KCN that was sensitive to *n*-propyl gallate. The O<sub>2</sub> concentration in air-saturated water at 28°C was assumed to be 253 μM and dry weight was determined as described below.

### Respiratory Characteristics of Isolated Mitochondria

Washed mitochondria were isolated under cold conditions from 4 × 200 mL of suspension cells. Cells were collected onto qualitative filter paper (VWR Canlab, Mississauga, ON, Canada) by vacuum filtration and washed briefly with ice-cold water. Cells were then scraped into a cooled commercial blender to which 300 mL of ice-cold homogenization medium was added. The homogenization medium consisted of 350 mM mannitol, 30 mM 3-(*N*-morpholino) propanesulfonic acid (pH 7.5), 1 mM EDTA, 10 mM Cys,

0.6% (w/v) polyvinylpyrrolidone, and 0.2% (w/v) bovine serum albumin. Cells were then disrupted by four pulses (of 3-s duration each) of the blender at maximum speed, and the homogenate was immediately filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego). The filtrate was centrifuged at low speed (3,850g, 2 min, 4°C), and the supernatant was then centrifuged at higher speed (20,600g, 10 min, 4°C) to pellet the mitochondria. The pellet was then carefully suspended in wash medium consisting of 300 mM mannitol, 20 mM 3-(*N*-morpholino) propane-sulfonic acid (pH 7.2), 1 mM EDTA, and 0.2% (w/v) bovine serum albumin. The sample was then centrifuged at low speed (3,850g, 2 min, 4°C), and the supernatant was centrifuged at high speed (20,600g, 10 min, 4°C) to pellet the mitochondria. The pellet was then suspended in a small volume of wash medium.

Immediately after isolation, O<sub>2</sub> uptake by mitochondria (adjusted to 0.1–0.25 mg protein mL<sup>-1</sup> in reaction medium) was measured in an oxygen electrode cuvette (as described above) at 28°C. The reaction medium contained 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 7.2), 250 mM Suc, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% (w/v) bovine serum albumin, and 0.1 mM each of NAD, NADP, ATP, and thiamine pyrophosphate. To initiate a maximal rate of electron transport, a combination of substrates consisting of 2 mM ADP, 2 mM NADH, and 10 mM each of succinate, malate, and Glu were added. Pyruvate (1 mM) and dithiothreitol (10 mM) were also present to ensure complete activation of AOX (Vanlerberghe et al., 1998). Under these assay conditions, mitochondrial respiration is defined as the O<sub>2</sub> uptake in the absence of any inhibitors minus any residual respiration (O<sub>2</sub> uptake in the presence of both KCN and *n*-propyl gallate). Mitochondrial cyt pathway capacity is defined as the O<sub>2</sub> uptake sensitive to 1 mM KCN in the presence of 20 μM *n*-propyl gallate. Mitochondrial AOX pathway capacity is defined as the O<sub>2</sub> uptake sensitive to 20 μM *n*-propyl gallate in the presence of 1 mM KCN. Stock solutions of pyruvate, NADH, and dithiothreitol were made fresh the day of use, whereas other components were stored frozen at -80°C. The O<sub>2</sub> concentration in air-saturated water at 28°C was assumed to be 253 μM and protein concentration was determined as described below.

### Protein Analysis of Mitochondria

Reducing SDS-PAGE was performed as previously described (Vanlerberghe et al., 1998). Monoclonal antibodies recognizing AOX (Elthon et al., 1989), cox II (a gift from Dr. A. Tzagaloff, Columbia University, New York), and cyt *c* (Pharming Canada, Mississauga, ON, Canada) were each used at a dilution of 1:2,000. Antibody detection was performed using the Super-signal West Pico Chemiluminescent detection system, according to the manufacturer's instructions (Pierce, Rockford, IL). Each antibody recognized a single prominent band of the expected size. A prestained broad range protein marker (New England Biolabs, Mississauga, ON, Canada) was used to estimate apparent molecular weights.

### DNA Isolation and Analysis

Isolation of genomic DNA was based on a method described by Mettler (1987). Fresh sampled cells (approximately 0.3 g dry weight) were disrupted with a mortar and pestle in 2 volumes of homogenization buffer consisting of 250 mM Suc, 1% (w/v) sarkosyl, 50 mM NaCl, 20 mM EDTA, and 50 mM tris-(hydroxymethyl) aminomethane (pH 8). The homogenate was incubated at room temperature for 30 min, after which an equal volume of phenol [equilibrated with a solution of 0.5 M NaCl and 100 mM tris-(hydroxymethyl) aminomethane, pH 8] was added. The mixture was mixed thoroughly and centrifuged (9,200g, 5 min, 4°C) in a swinging bucket rotor. The top aqueous layer was collected and mixed with another equal volume of equilibrated phenol. After another centrifugation, the aqueous phase was collected and mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold 100% (v/v) ethanol. The sample was left overnight at -20°C and then centrifuged (9,150g, 15 min, 4°C). The pellet of DNA was washed with 70% (v/v) ethanol, dried briefly, and suspended in a solution containing 10 mM tris-(hydroxymethyl) aminomethane (pH 8) and 1 mM EDTA. The sample was then treated with 10 μg mL<sup>-1</sup> DNase-free RNaseA at 37°C for 40 min. DNA was subsequently quantified using the agarose plate gel method using salmon sperm DNA as a standard (Sambrook and Russell, 2001). Then, 4 μg of DNA was separated on a 2% (w/v) agarose gel containing ethidium bromide, visualized on a UV-transilluminator, and

photographed. A 100-bp DNA ladder (New England Biolabs) was also run on the gel.

### Other Methods

Cell viability was determined by microscopic observation of cells treated with Evans blue, which accumulates in dead cells as a blue protoplasmic stain (Baker and Mock, 1994). Typically, 500 to 750 cells were scored to establish viability of a culture. To determine cell dry weight, an aliquot of the cell culture was washed twice with water, frozen, and lyophilized. Protein concentration was determined by a modified Lowry method (Larson et al., 1986). Statistical analyses were performed using Prism 3 (GraphPad Software, San Diego).

### ACKNOWLEDGMENTS

We thank Dr. Sasan Amirsadeghi, David Couto, Sandi H. Ordov, Mark Sandico, and Justine Y.H. Yip for their contributions to this work.

Received March 1, 2002; returned for revision April 9, 2002; accepted April 26, 2002.

### LITERATURE CITED

- Adams JM, Corey S (2001) Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci* **26**: 61–66
- Alvarez ME (2000) Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant Mol Biol* **44**: 429–442
- Amor Y, Chevion M, Levine A (2000) Anoxia pretreatment protects soybean cells against H<sub>2</sub>O<sub>2</sub>-induced cell death: possible involvement of peroxidases and of alternative oxidase. *FEBS Lett* **477**: 175–180
- Arpagaus S, Rawlyer A, Braendle R (2002) Occurrence and characteristics of the mitochondrial permeability transition in plants. *J Biol Chem* **277**: 1780–1787
- Assaad FF (2001) Of weeds and men: what genomes teach us about plant cell biology. *Curr Opin Plant Biol* **4**: 478–487
- Baker CJ, Mock NM (1994) An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. *Plant Cell Tissue Org Cult* **39**: 7–12
- Balk J, Leaver CJ (2001) The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome *c* release. *Plant Cell* **13**: 1803–1818
- Beers EP, McDowell JM (2001) Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr Opin Plant Biol* **4**: 561–567
- Bethke PC, Jones RL (2001) Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. *Plant J* **25**: 19–29
- Bratton SB, Cohen GM (2001) Apoptotic death sensor: an organelle's alter ego? *Trends Pharmacol Sci* **22**: 306–315
- Desagher S, Martinou J-C (2000) Mitochondria as the central point of apoptosis. *Trends Cell Biol* **10**: 369–377
- Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of plant mitochondria. *Plant Physiol* **89**: 1311–1317
- Green D, Kroemer G (1998) The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol* **8**: 267–271
- Hoerberichts FA, Orzaez D, van der Plas LHW, Woltering EJ (2001) Changes in gene expression during programmed cell death in tomato cell suspensions. *Plant Mol Biol* **45**: 641–654
- Jabs T (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem Pharmacol* **57**: 231–245
- Jones A (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? *Trends Plant Sci* **5**: 225–230
- Kaufmann SH, Hengartner MO (2001) Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* **11**: 526–534
- Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P et al. (2000) Nitric oxide and salicylic acid signaling in plant defense. *Proc Natl Acad Sci USA* **97**: 8849–8855
- Kokoszka JE, Coskun P, Esposito LA, Wallace DC (2001) Increased mitochondrial oxidative stress in the Sod2 (+/-) mouse results in the age-

- related decline of mitochondrial function culminating in increased apoptosis. *Proc Natl Acad Sci USA* **98**: 2278–2283
- Korthout HAAJ, Berecki G, Bruin W, van Duijn B, Wang M** (2000) The presence and subcellular localization of caspase 3-like proteinases in plant cells. *FEBS Lett* **475**: 139–144
- Kowaltowski AJ, Castilho RF, Vercesi AE** (2001) Mitochondrial permeability transition and oxidative stress. *FEBS Lett* **495**: 12–15
- Lacomme C, Roby D** (1999) Identification of new early markers of the hypersensitive response in *Arabidopsis thaliana*. *FEBS Lett* **459**: 149–153
- Lacomme C, Santa Cruz S** (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proc Natl Acad Sci USA* **96**: 7956–7961
- Lam E, Kato N, Lawton M** (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**: 848–853
- Larson E, Howlett B, Jagendorf A** (1986) Artificial reductant enhancement of the Lowry method for protein determination. *Anal Biochem* **155**: 243–248
- Li YM, Casida JE** (1992) Cantharidin-binding protein: identification as protein phosphatase 2A. *Proc Natl Acad Sci USA* **89**: 11867–11870
- Linsmaier EM, Skoog F** (1965) Organic growth factor requirement of tobacco tissue cultures. *Physiol Plant* **18**: 100–127
- Matsuyama S, Reed JC** (2000) Mitochondria-dependent apoptosis and cellular pH regulation. *Cell Death Diff* **7**: 1155–1165
- Maxwell DP, Wang Y, McIntosh L** (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci USA* **96**: 8271–8276
- Mettler IJ** (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. *Plant Mol Biol Rep* **5**: 346–349
- Miller AH, Day DA** (1996) Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. *FEBS Lett* **398**: 155–158
- Mitsuhashi I, Malik KA, Miura M, Ohashi Y** (1999) Animal cell-death suppressors Bcl-x<sub>L</sub> and Ced-9 inhibit cell death in tobacco plants. *Curr Biol* **9**: 775–778
- Mootha VK, Wei MC, Buttle KF, Scorrano L, Panoutsakopoulou V, Mannella CA, Korsmeyer SJ** (2001) A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. *EMBO J* **20**: 661–671
- Morris K, Mackerness SAH, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V** (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J* **23**: 677–685
- Parsons HL, Yip JYH, Vanlerberghe GC** (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. *Plant Physiol* **121**: 1309–1320
- Petrosillo G, Ruggiero FM, Pistolesi M, Paradies G** (2001) Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation: possible role in the apoptosis. *FEBS Lett* **509**: 435–438
- Pique M, Barragan M, Dalmau M, Bellosillo B, Pons G, Gil J** (2000) Aspirin induces apoptosis through mitochondrial cytochrome c release. *FEBS Lett* **480**: 193–196
- Pontier D, Gan S, Amasino RM, Roby D, Lam E** (1999) Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Mol Biol* **39**: 1243–1255
- Purvis AC** (1997) Role of the alternative oxidase in limiting superoxide production by plant mitochondria. *Physiol Plant* **100**: 165–170
- Rakwal R, Shii K, Agrawal GK, Yonekura M** (2001) Protein phosphatase inhibitors activate defense responses in rice (*Oryza sativa*) leaves. *Physiol Plant* **111**: 151–157
- Rao MV, Davis KR** (2001) The physiology of ozone induced cell death. *Planta* **213**: 682–690
- Ryerson DE, Heath MC** (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* **8**: 393–402
- Sambrook J, Russell DW** (2001) Commonly used techniques in molecular cloning. *In* Molecular Cloning: A Laboratory Manual, Vol 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp A8.24
- Sun Y-L, Zhao Y, Hong X, Zhai Z-H** (1999) Cytochrome c release and caspase activation during menadione-induced apoptosis in plants. *FEBS Lett* **462**: 317–321
- Suzuki K, Yano A, Shinshi H** (1999) Slow and prolonged activation of the p47 protein kinase during hypersensitive cell death in a culture of tobacco cells. *Plant Physiol* **119**: 1465–1472
- Vanlerberghe GC, McIntosh L, Yip JYH** (1998) Molecular localization of a redox-modulated process regulating plant mitochondrial electron transport. *Plant Cell* **10**: 1551–1560
- Vanlerberghe GC, Ordog SH** (2002) Alternative oxidase: integrating carbon metabolism and electron transport in plant respiration. *In* CH Foyer, G Noctor, eds, *Advances in Photosynthesis and Respiration. Photosynthetic Nitrogen Assimilation and Associated Carbon Metabolism*. Kluwer Academic Publishers, Dordrecht, The Netherlands (in press)
- Vanlerberghe GC, Vanlerberghe AE, McIntosh L** (1994) Molecular genetic alteration of plant respiration: silencing and overexpression of alternative oxidase in transgenic tobacco. *Plant Physiol* **106**: 1503–1510
- Voehringer DW, Hirschberg DL, Xiao J, Roederer M, Lock CB, Herzenberg LA, Steinman L, Herzenberg LA** (2000) Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. *Proc Natl Acad Sci USA* **97**: 2680–2685
- Xie Z, Chen Z** (1999) Salicylic acid induces rapid inhibition of mitochondrial electron transport and oxidative phosphorylation in tobacco cells. *Plant Physiol* **120**: 217–225
- Yip JYH, Vanlerberghe GC** (2001) Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake. *Physiol Plant* **112**: 327–333